Schistosoma mansoni eggs modulate the timing of granuloma formation to facilitate parasite maturation and release

Kevin K. Takaki[1], Gabriel Rinaldi[2], Matthew Berriman[2], Antonio J. Pagán[1*], Lalita Ramakrishnan[1*]

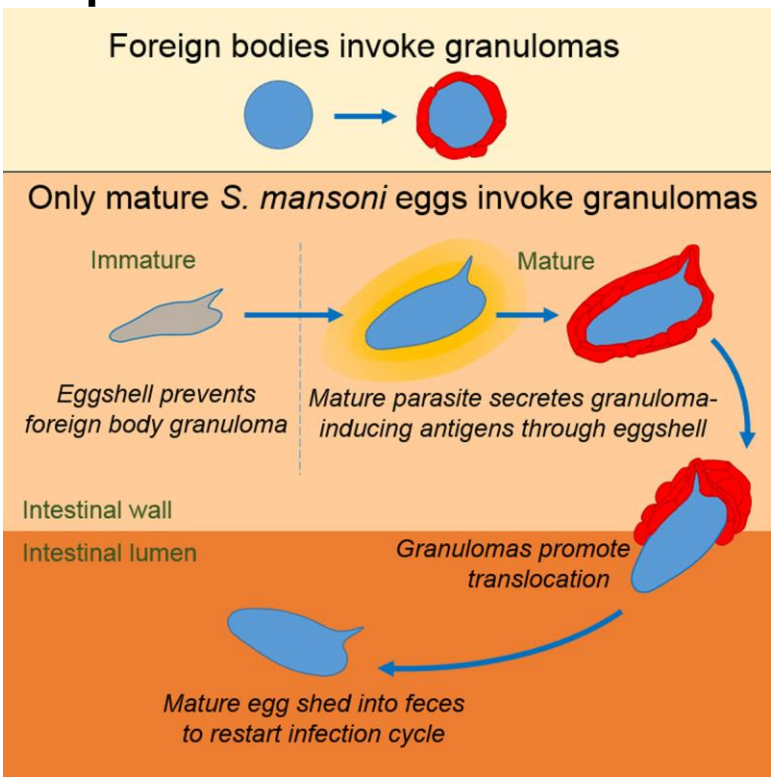
[1]Molecular Immunity Unit, Department of Medicine, University of Cambridge, MRC Laboratory of Molecular Biology, Cambridge, CB2 0QH, UK

[2]Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, CB10 1SA, UK

*Correspondence to: ap825@cam.ac.uk (A.J.P.); lr404@cam.ac.uk (L.R.)

Lead contact: lr404@cam.ac.uk (L.R.)

Graphical Abstract



Highlights

- Foreign bodies are walled off by immune structures called granulomas
- *Schistosoma mansoni* eggshells prevent granulomas forming around immature parasites
- Secreted antigens from mature parasites induce granulomas that promote egg shedding
- *S. mansoni* modulates granuloma formation to selectively shed mature eggs into feces

SUMMARY

Schistosome eggs provoke formation of granulomas around them. For the host, the granulomatous response can be both protective and pathological. Granulomas are also postulated to facilitate parasite egg extrusion through the gut lumen, a necessary step for transmission. Using zebrafish larvae, we find that mature *Schistosoma mansoni* eggs recruit macrophages, which form granulomas within days. Inert egg-sized beads also rapidly induce granulomas through a foreign body response. Strikingly, immature *S. mansoni* eggs do not recruit macrophages. We distinguish mature and immature eggs via size analysis and find that only mature *S. mansoni* eggs are shed into the feces of infected mice. Importantly, analysis of egg sizes from previously reported data shows that humans also shed mature eggs only. Our findings support the model that the immunologically inert eggshell inhibits granuloma formation long enough for the parasite to mature. Then parasite antigens secreted through the eggshell trigger granulomas, facilitating egg extrusion.

INTRODUCTION

Human schistosomiasis, caused by parasitic flatworms of the genus *Schistosoma*, affects more than 200 million people worldwide (WHO, 2019). Adult schistosomes live in the mesenteric venules of their definitive hosts, humans, where they produce eggs that are shed into the environment through feces or urine, depending on the schistosome species (Colley and Secor, 2014). Upon reaching fresh water, the eggs hatch releasing free swimming larvae, miracidia, that can then infect their intermediate snail hosts (Colley and Secor, 2014). In the snails, they reproduce asexually and mature to produce cercarial larvae, which are released into the water, and infect humans by penetrating the skin (Colley and Secor, 2014). In the case of *Schistosoma mansoni*, the most studied and geographically widespread species, the egg-laying adult pair resides in the mesenteric venous plexus. Upon maturation in the liver, the female and male adult worms pair up and migrate via the portal system to the mesenteric venules where they produce eggs (Nation et al., 2020). The eggs are shed by translocation through the venule and then the intestinal wall into the feces; however, many become lodged in the intestinal wall or the liver (Hams et al., 2013; McManus et al., 2018; Nation et al., 2020; Schwartz and Fallon, 2018).

As the egg matures, it secretes antigens that provoke the formation of a granuloma - an organized aggregate of macrophages and other immune cells - around it (Ashton et al., 2001; Boros and Warren, 1970; Chiu and Chensue, 2002; Jurberg et al., 2009). For the host, the granuloma may play a dual function - both protective and pathogenic (Hams et al., 2013). On the one hand, it may protect the host by sequestering toxic egg antigens, and by preventing translocation of bacteria from the intestinal lumen into the tissues as the egg breaches the intestinal wall to exit the host (Costain et al., 2018; Hams et al., 2013; Pagan and Ramakrishnan, 2018; Schwartz and Fallon, 2018). On the other hand, the chronic granulomas around tissue-

trapped eggs, particularly those in the liver, are the principal drivers of disease pathogenesis and morbidity (Hams et al., 2013; Pagan and Ramakrishnan, 2018). The chronic *Schistosoma* granuloma has a complex cellular composition with an abundance of myeloid cells, lymphocytes, eosinophils, and fibroblasts that act in concert to cause tissue pathology (Hams et al., 2013; Pagan and Ramakrishnan, 2018). The fibrogenic granulomatous response to the liver-trapped eggs causes damaging periportal fibrosis leading to portal hypertension and the development of esophageal varices which can rupture leading to internal bleeding and death (Colley and Secor, 2014; Pagan and Ramakrishnan, 2018).

While the granuloma's role has mainly been studied from a host centric view, it has also been hypothesized that the early granuloma is critical for the parasite's life cycle by facilitating the translocation of the eggs from the vasculature to the intestines and then into the feces for transmission to a new host (Dunne et al., 1983; Hams et al., 2013; Schwartz and Fallon, 2018). Because insights into the *Schistosoma* granuloma have been derived from single time point histologic studies of human clinical samples and animal models - hamsters, mice and monkeys (Cheever et al., 2002; Hutchison, 1928), its role in translocation is understudied. The optical transparency of the zebrafish larva has enabled detailing of the early events of tuberculous granuloma formation in real-time using non-invasive, high resolution, serial intravital microscopy (Pagan and Ramakrishnan, 2018; Ramakrishnan, 2020; Takaki et al., 2013). Here, we have used the zebrafish larva to detail the events of early granuloma formation to *S. mansoni* eggs. We find that macrophage-dense epithelioid granulomas form rapidly around mature eggs. In striking contrast, we find that immature eggs are immunologically silent, failing to provoke even minimal macrophage recruitment. Given that inert beads induce epithelioid granulomas, this finding provides insight into how the egg might actively manipulate the timing of granuloma

formation so as to prevent immune destruction or premature extrusion from the host. This idea is supported by our findings that *S. mansoni*-infected mice have both mature and immature eggs in their liver and intestinal wall but shed only mature eggs into the intestinal lumen.

RESULTS

S. mansoni eggs induce epithelioid granuloma formation in the context of innate immunity

To study *Schistosoma* granulomas we used the zebrafish hindbrain ventricle (HBV), an epithelium-lined cavity to which phagocytes are recruited in response to chemokines and bacteria (Cambier et al., 2017; Cambier et al., 2014; Takaki et al., 2013; Yang et al., 2012)(Figure 1A). It has previously been shown that beads coated with *S. mansoni* soluble egg antigens (SEA) injected intravenously into mice get deposited in the lung where they induce macrophage recruitment and aggregation around them (Boros and Warren, 1971; Chiu et al., 2004). Using transgenic zebrafish with red fluorescent macrophages, we found that injection of SEA into the HBV induced macrophage recruitment within six hours (Figure 1B). Next, we implanted *S. mansoni* eggs into the HBV. Because the mature egg is relatively large (>50 μm diameter), we used a large bore borosilicate needle that allowed us to make an incision, grasp the egg and implant it into the HBV cavity in rapid succession (Figure S1, Movie S1 and Methods, Figure 1C). Implantation of the eggs had no deleterious effect on larval survival; larvae implanted with either one or two eggs had a survival rate of 98%-100% at 5 days post-implantation (dpi), identical to the mock-implanted control group (n=50 per group). Implantation also did not change larval swimming behaviors or responses to tactile stimuli.

We examined macrophage responses to the egg at 5 dpi. Eight independent experiments showed a consistent pattern of varying levels of macrophage recruitment: some eggs (32%, range 17 to 44%) had minimal macrophage recruitment with 0-6 macrophages found in contact with

the egg (Figure 1D and 1E and Supplementary Table 1). The majority (69%, range 56 to 83%) elicited robust macrophage recruitment with 41% (range 11 to 67%) having several isolated macrophages or small clusters of macrophages in contact with them and 28% (0 to 45%) eliciting organized granulomas that had either partially or fully enveloped them (Figure 1D and 1E and Supplementary Table 1).

To determine the macrophage recruitment events leading to granuloma formation, we imaged nine implanted eggs sequentially over seven days, and then analyzed retrospectively the progression of recruitment in the three that had formed granulomas (Figure 2A and Figure S2). For the egg shown in Figure 2A, by 1 dpi, macrophages had arrived in response to the egg and were in contact with it (Figure 2A; Movie S2). By 3 dpi, macrophages had formed loose aggregates on one part of the egg (Figure 2A), an intermediate stage that is likely to represent a transient transition to granuloma formation as it was not seen in our 5 dpi single timepoint analyses. By 5 dpi, an organized granuloma partially covering the egg was apparent, which had expanded to encapsulate the entire egg by 7 dpi (Figure 2A; Movie S2). In the remaining two eggs that elicited granulomas, one had a similar sequence of events except that the granuloma which formed by 5 dpi had still not enveloped the egg completely at 7 dpi (Figure S2A). The other egg had already formed a small partial granuloma by 3 dpi but could not be monitored further owing to failure to recover the animal following imaging on this day (S2B). Thus, the sequence of events leading to granuloma formation seemed consistent in all cases.

Likewise, in all three cases, even the partial granulomas had macrophages which appeared confluent with indistinct intercellular boundaries, suggesting they had already undergone the characteristic epithelioid transformation associated with mature *Schistosoma* granulomas (Moore et al., 1977; Von Lichtenberg et al., 1973)(Figure 2A and Figure S2). To confirm this, we

identified 8 eggs that had elicited partial or complete granulomas and assessed these for epithelioid transformation using immunofluorescence staining for E-cadherin, the expression of which is its cardinal feature (Cronan et al., 2016). All 8 eggs had E-cadherin staining, confirming that they had undergone epithelioid transformation as exemplified by Figure 2B and Movie S2.

In mammals, *S. mansoni* eggs invoke macrophage-rich granulomas with very few neutrophils in contrast to *S. japonicum* eggs, which recruit both macrophages and neutrophils (Chensue et al., 1995; Moore et al., 1977; Swartz et al., 2006; Von Lichtenberg et al., 1973). Likewise, we found that in the zebrafish, granulomas forming to *S. mansoni* eggs contained very few neutrophils (Figure 2C and 2D). In contrast, similarly-sized *Mycobacterium marinum* granulomas all contained neutrophils as expected (Figure 2C and 2D) (Yang et al., 2012). This pattern was established at the onset of egg implantation with the recruitment of macrophages but not neutrophils at 6 hours post-implantation (hpi), whereas the Gram-negative bacterium *Pseudomonas aeruginosa* recruited both types of cells, as expected (Figure 2E)(Yang et al., 2012). The lack of neutrophil recruitment has been attributed to the egg-secreted, interleukin-8 neutralizing *S. mansoni* chemokine binding protein (smCKBP), more commonly known as alpha-1 (Smith et al., 2005). Accordingly, the injection of SEA recruited macrophages but not neutrophils, in contrast to *P. aeruginosa* which recruited both (Figure 2F and 2G).

Next, we asked if the miracidium could survive within an epithelioid granuloma. We imaged individual eggs containing mature miracidia within organized granulomas at 5 dpi, and found they were still alive; the miracidium could be seen moving within the eggshell (Figure S3A; Movie S3). E-cadherin staining immediately after imaging confirmed that the granuloma macrophages had indeed undergone epithelioid transformation (Figure S3B). We also saw that in those cases where the eggshell had ruptured either during or after implantation, macrophages had

entered into the eggshell and destroyed the miracidium (Figure S3C; Movie S3). These findings were consistent with those in mammals showing that the intact eggshell protects the miracidium against destruction by host macrophages (Bunnag et al., 1986; Hutchison, 1928; Von Lichtenberg et al., 1973). Further confirming this, miracidia implanted after removal from the egg rapidly recruited macrophages that destroyed them (Figure S3D).

In sum, we found that the key features of early mammalian responses to *S. mansoni* eggs are replicated in the zebrafish: selective macrophage recruitment to form bona fide epithelioid granulomas within days, which formed in the sole context of innate immunity. Our findings highlight that the miracidium tolerates granuloma formation as long as the eggshell is intact, a critical aspect of the *Schistosoma* life cycle that depends on granulomas to enhance egg extrusion from the host. These granulomas most closely resemble intestinal granulomas in mice, which comprise mostly macrophages with fewer lymphocytes and eosinophils (Weinstock and Boros, 1983).

Immature *S. mansoni* eggs do not induce macrophage recruitment or granuloma formation

The egg matures six days after it is fertilized at which point it begins to secrete antigens (Ashton et al., 2001; Jurberg et al., 2009; Mann et al., 2011; Michaels and Prata, 1968). Accordingly, only viable mature eggs are found to induce granulomas (Jurberg et al., 2009; Von Lichtenberg et al., 1973). We sorted immature and mature eggs based on their size and appearance (Figure S4A)(Jurberg et al., 2009). None of the immature eggs had reached maturity by 5 dpi and importantly all invoked only minimal macrophage recruitment (Figure 3A and 3B). To corroborate this result, we implanted in vitro laid eggs at 2 and 6 days post-fertilization in which the developmental stages were synchronized so that the two day eggs were immature and

the six day eggs mature (Figure S4B). Again, the majority of the six day old mature eggs induced macrophage recruitment, including granuloma formation, whereas the two-day eggs elicited only minimal macrophage recruitment (Figure 3C). These results were consistent with antigens secreted from the mature egg being the trigger for granuloma formation (Ashton et al., 2001; Boros and Warren, 1970; Chiu and Chensue, 2002). To test this, we asked if dead eggs elicited a macrophage response. Freshly heat-killed eggs produced fewer granulomas than live eggs (Figure 3D). This finding is consistent with prior observations that some egg antigens are heat stable and that heat-killed eggs retain a thin layer of antigens which can induce granulomas, albeit less than living eggs (Freedman and Ottesen, 1988; Klaver et al., 2015; Von, 1964). Accordingly, we found that eggs that had been killed by storage at 4°C for 12 months (old dead eggs) so as to potentially inactivate all their antigens did not induce granulomas, and only a minority (10%) recruited any macrophages at all (Figure 3E).

We next asked if immature and dead eggs, although failing to form granulomas, could still induce early transient macrophage recruitment. At 6 hours post-implantation, immature, heat-killed, and old dead eggs all recruited fewer macrophages than live mature eggs (Figure 3F-3I). These findings suggested that mature egg antigens enhance macrophage recruitment from the earliest stages, and subsequently activate the recruited macrophages to form the granuloma.

Finally, we found that if we ruptured immature eggs prior to implantation, they rapidly recruited macrophages (Figure 3J and 3K). Similar to the case with ruptured mature eggs, these macrophages entered the ruptured immature egg and killed the embryo (Figure 3J and data not shown). Together these results suggest that while the exposed embryo and fully-mature miracidium elicit macrophage recruitment similarly, the intact egg at the two stages is

fundamentally different in its ability to recruit macrophages, the initial step that is required for granuloma formation.

To ask if egg antigen secretion was also required for the subsequent steps of macrophage aggregation into granulomas, we implanted an immature egg together with a mature egg in each animal. If mature egg antigens were required only to recruit macrophages to the egg, then the presence of the mature egg should recruit macrophages to the vicinity of the immature egg, allowing granulomas to form. In both instances, macrophages were recruited to and settled on the mature egg, with hardly any on the adjacent immature egg (Figure 3L). Thus, macrophage recruitment in response to the presence of egg antigens in the vicinity of the immature egg is not sufficient to induce macrophage adherence and granuloma formation. Rather, egg-intrinsic antigen is required for both macrophage recruitment and adherence to the egg with subsequent granuloma formation.

The immature Schistosoma egg evades foreign body granuloma formation

Our findings were consistent with macrophage recruitment occurring only in response to antigens secreted from the mature egg rather than to the eggshell itself. Granulomas form in response to inert foreign bodies (Pagan and Ramakrishnan, 2018), so why would the eggshell not induce a foreign body granuloma? We considered three possibilities. First, that it was too small to invoke a foreign body response; this seemed unlikely as very small inert particles, e.g. a tiny thorn, can provoke a robust foreign body response (Pagan and Ramakrishnan, 2018). Second, that the mechanisms to form foreign body granulomas were not yet operant in the developing zebrafish larvae; this too seemed unlikely given that the foreign body granuloma response is evolutionarily ancient, and epithelioid granulomas form in response to foreign bodies in invertebrates (Pagan and Ramakrishnan, 2018). Third, that the immature schistosome egg has

specific mechanisms to evade foreign body granuloma formation. To distinguish between these possibilities, we implanted beads of three different chemically inert materials of the same size as the schistosome egg (Table S2). We chose sepharose, which is hydrophilic, and polystyrene and polyethylene, which are hydrophobic. All recruited macrophages within six hours (Figure 4A and 4B). By five days, epithelioid granulomas had surrounded most of the sepharose and polystyrene beads (Figure 4C-4E). The polyethylene beads were less granuloma inciting, with only 11% inducing bona fide granulomas, and most of the remaining beads failing to retain recruited macrophages (Figure 4C and 4D). However, even this weaker response was more robust than that of the immature eggs, which did not even transiently recruit macrophages. We confirmed these findings with a head-on comparison of macrophage recruitment and granuloma formation in response to immature eggs or similarly sized polystyrene beads in the same experiment (Table S2). Again, the polystyrene beads recruited macrophages by six hours and formed granulomas by five days, whereas the immature eggs did neither (Figure 4F and 4G). This result suggested that the immature egg specifically avoids being recognized as a foreign body. This could be because the immature egg secretes a specific product to inhibit macrophage recruitment, or that the eggshell is immunologically inert. To distinguish between these possibilities, we implanted an immature egg and a polystyrene bead adjacent to each other in the same animal. In every case, at six hours, macrophages were recruited only to the bead and not to the egg (Figure 4H and 4I). By five days post-implantation, granulomas had formed around the beads but none of the immature eggs (Figure 4J). These results support the idea that the eggshell evolved to be immunologically inert so as to evade the ubiquitous foreign body granulomatous response.

Only mature eggs translocate into the intestinal lumen of *S. mansoni*-infected mice and humans

The observation that immature eggs, unlike mature eggs, are immunologically silent, led us to hypothesize, as P.D. Ashton et al. did before (Ashton et al., 2001), that timing granuloma formation to egg maturation prevents the expulsion of immature eggs while they are still dependent on the absorption of nutrients from the host for development. Moreover, only a mature miracidium can survive in the aquatic environment and invade its snail host. If our hypothesis were true, we would expect to find in *S. mansoni*-infected mice, an enrichment of mature eggs in the intestinal lumen as compared to intestinal wall and liver. To test this prediction, mice were naturally infected with *S. mansoni* by cutaneous exposure to cercaria, and at 6 weeks post-infection, eggs were analyzed from liver, intestinal tissue, and small and large intestinal luminal content (feces). We quantified and categorized eggs as mature or immature by size and morphology (Jurberg et al., 2009)(Figure S4 and S5). As a test of our scoring accuracy, we then measured the size of the eggs and confirmed that our visual inspection had correctly separated the immature and mature eggs (Figure S5). We next assessed the distribution of mature and immature eggs for each collection site in each mouse. We found that while the liver and the intestinal tissue contained roughly equal proportions of both immature and mature eggs (Figure 5A-D), hardly any immature eggs were found in the small intestinal lumen (6% average for all six animals; Figure 5E). Moreover, only 2 out of 11 immature eggs were at the very early stage of development, with the remaining ones nearing maturity (2009 Jurberg)(Figure 5E-G). All eggs scored from the lumen of the large intestines (feces) were morphologically mature and contained fully mature miracidia (Figure 5H and 5I). Statistical analysis of the pooled data from four mice

confirmed an enrichment of mature eggs in the lumen of the small and large intestines (Figure 5J). These results confirmed that virtually all eggs shed by infected mice are mature.

Do humans also shed only mature eggs? We were unable to find a direct answer to this question in the literature. However, we found a paper that had assessed the length and width of 30 eggs shed in the feces of *S. mansoni*-infected humans (Martinez., 1916). Because we found that immature and mature eggs differ in size with immature eggs being much smaller (Figure S4 and S5)(Ashton et al., 2001), we were in a position to determine if the eggs shed by humans were mature or immature. We plotted the sizes of the eggs shed in human feces alongside the eggs from the mouse intestinal wall and lumen and found that all of the human eggs were in the mature egg size range (Figure 5K). Thus, humans also shed only mature eggs.

These results support the hypothesis that the timing of granuloma formation and subsequent egg expulsion is modulated so as to prevent premature expulsion of immature eggs, which would be terminal for the parasite were it to occur.

DISCUSSION

Research on *S. mansoni* granulomas has focused mainly on the organ-damaging fibrosis that ensues from granulomas forming around tissue-lodged eggs (Colley and Secor, 2014). Yet most *S. mansoni*-infected individuals are either asymptomatic or only mildly symptomatic (Hams et al., 2013), possibly because their granulomatous response is more tempered. These individuals shed parasite eggs, highlighting that disease per se does not benefit the parasite's evolutionary survival. Rather, as in the case with many infectious diseases, human disease represents collateral damage stemming from the host-pathogen interaction, harming the host with little benefit to the pathogen (Relman et al., 2020). On the other hand, early granuloma formation

in appropriate anatomical locations is thought to benefit both host and parasite for the same reason, expelling the parasite egg from the human host so as to enable it to continue its life cycle in its intermediate snail host (Dunne et al., 1983; Hams et al., 2013). While this idea is appreciated, it has been difficult to study extensively because of experimental limitations. Early or asymptomatic human infection seldom presents itself for study, and existing animal models are less suitable for the study of early granuloma-associated pathology.

This work explores the earliest steps of *Schistosoma* granuloma formation that have not been captured in existing animal models. We show that as is the case with mycobacterial granulomas, bona fide epithelioid granulomas form in response to the Schistosoma egg in the sole context of innate immunity (Cronan et al., 2016; Davis et al., 2002). This should not be surprising given that epithelioid granulomas form in multiple invertebrate species in response to retained foreign bodies or even their own dead eggs (Pagan and Ramakrishnan, 2018). Yet, there has been at best a limited appreciation that adaptive immunity is not required for the formation of such an organized structure in the context of infectious granulomas, and indeed, the emphasis of schistosomiasis research on the late-stage granuloma has caused the focus to be on how the granuloma is modulated by adaptive immunity to become pathogenic (Hams et al., 2013; Pagan and Ramakrishnan, 2018). Given that Schistosoma eggs begin to be shed into the feces within days following maturation (deWalick et al., 2012), egg shedding must occur even in the absence of adaptive immunity and is likely promoted by these innate epithelioid granulomas. Our finding of the rapid epithelioid transformation of the granuloma also has relevance for granuloma-induced transmission later in infection when adaptive immunity is operant. Moreover, intestinal granulomas, the ones that extrude the eggs, are smaller than those in the liver, with a paucity of the lymphocytes and eosinophils that characterize liver granulomas (Weinstock and Boros, 1983).

The rapid epithelioid transformation of the *Schistosoma* granuloma may help it to more efficiently extrude the eggs and hence propagate the parasite.

We have also gained understanding of the mechanics of early granuloma formation. Broadly speaking, granuloma formation in response to the mature egg proceeds in two discrete steps. In the first step, macrophages are attracted to secreted parasite antigens, and upon contact with the egg, appear to gain a chemotactic activity that outstrips that of the egg. This results in the subsequent macrophages being recruited to the existing macrophages forming a tight, aggregate that then pulls itself together to encapsulate the egg. It is noteworthy that epithelioid transformation precedes the complete covering of the egg, highlighting that this specialized macrophage transformation (Pagan and Ramakrishnan, 2018) constitutes an early response.

While these new details on how granulomas form around mature eggs are thought-provoking, more striking is the lack of even minimal macrophage recruitment by the immature egg. Given that like-sized beads recruit macrophages robustly and induce epithelioid granulomas, this finding reveals further nuance to the exploitation of the granuloma by the parasite. Not only must the parasite turn on granuloma formation through secretion of antigens, but it must also prevent the granuloma from forming too soon. Premature granuloma formation may thwart granuloma for one of two reasons. The egg is laid into the blood stream, and needs to reach the wall of the blood vessel from which it extravasates and then penetrates the gut wall to be shed (deWalick et al., 2012). This process takes at least six days, perfectly synchronized with the time it takes for the miracidium to mature (Michaels and Prata, 1968; Pellegrino et al., 1962). Perhaps, premature granuloma formation might encumber its passage to the intestinal wall. Second, premature extrusion would remove the egg from the human tissue environment that is conducive to its maturation (Ashton et al., 2001).

316 Prior work has noted that the granuloma-inducing secreted Schistosoma antigens are secreted
317 from the egg, rather than being incorporated into the eggshell, and that secretion occurs only
318 after egg maturation (Ashton et al., 2001; Schwartz and Fallon, 2018). This work adds the key
319 insight that the immunologically inert nature of the eggshell is a requisite counterpart of the
320 Schistosoma transmission strategy. Our ability to directly compare granuloma formation around
321 eggs and beads has been key to this insight. It will be interesting to determine how the eggshell
322 remains immunologically inert in the context of adaptive immunity, particularly because eggshell
323 proteins induce antibodies in humans (Dewalick et al., 2011; deWalick et al., 2012). Foreign
324 body granuloma formation is a major complication of implanted devices (Pagan and
325 Ramakrishnan, 2018). Identifying the chemical basis of the granuloma-silencing mechanism of
326 the eggshell may have therapeutic implications to design inert materials for medical implants that
327 alleviate this problem.
328
329 **AUTHOR CONTRIBUTIONS**
330 A.J.P. and L.R. conceived the research project. K.K.T., A.J.P. and L.R. conceived and designed
331 experiments and analyzed and interpreted data. K.K.T. performed the experiments. G.R. and
332 M.B. provided knowledge, insights, experimental guidance and help with data analysis and
333 interpretation. K.K.T and L.R. wrote the paper. K.K.T. made the figures. A.J.P., G.R. and M.B.
334 edited the paper.
335
336 **ACKNOWLEDGMENTS**
337 We thank P. Edelstein for advice, discussion, and identifying a key paper from 1916, S. Clare, C.
338 Brandt, K. Harcourt, L. Seymour and C. McCarthy for assistance and technical support with

339 animal infections and maintenance of the *S. mansoni* life cycle, P. Driguez and S. Buddenborg
340 for support with *S. mansoni* egg preparation, G. Schramm for SEA preparations, and R. Keeble
341 and N. Goodwin for zebrafish husbandry. This work was supported by Wellcome Trust core-
342 funding support to the Wellcome Sanger Institute (award number 206194) (G.R., M.B.) and NIH
343 MERIT award (R37 AI054503) and a Wellcome Trust Principal Research Fellowship (L.R.).

REFERENCES

Ashton, P.D., Harrop, R., Shah, B., and Wilson, R.A. (2001). The schistosome egg: development and secretions. Parasitology *122*, 329-338.

Boros, D.L., and Warren, K.S. (1970). Delayed hypersensitivity-type granuloma formation and dermal reaction induced and elicited by a soluble factor isolated from Schistosoma mansoni eggs. J Exp Med *132*, 488-507.

Boros, D.L., and Warren, K.S. (1971). Specific granulomatous hypersensitivity elicited by bentonite particles coated with soluble antigens from schistosome eggs and turcle bacilli. Nature *229*, 200-201.

Bunnag, T., Impand, P., and Sornmani, S. (1986). Schistosoma japonicum-like infection in Phichit province, northern Thailand: a case report. Southeast Asian J Trop Med Public Health *17*, 189-193.

Cambier, C.J., O'Leary, S.M., O'Sullivan, M.P., Keane, J., and Ramakrishnan, L. (2017). Phenolic Glycolipid Facilitates Mycobacterial Escape from Microbicidal Tissue-Resident Macrophages. Immunity *47*, 552-565 e554.

Cambier, C.J., Takaki, K.K., Larson, R.P., Hernandez, R.E., Tobin, D.M., Urdahl, K.B., Cosma, C.L., and Ramakrishnan, L. (2014). Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. Nature *505*, 218-222.

Cheever, A.W., Lenzi, J.A., Lenzi, H.L., and Andrade, Z.A. (2002). Experimental models of Schistosoma mansoni infection. Mem Inst Oswaldo Cruz *97*, 917-940.

Chensue, S.W., Warmington, K.S., Ruth, J.H., Lincoln, P., and Kunkel, S.L. (1995). Cytokine function during mycobacterial and schistosomal antigen-induced pulmonary granuloma

formation. Local and regional participation of IFN-gamma, IL-10, and TNF. J Immunol *154*, 5969-5976.

Chiu, B.C., and Chensue, S.W. (2002). Chemokine responses in schistosomal antigen-elicited granuloma formation. Parasite Immunol *24*, 285-294.

Chiu, B.C., Freeman, C.M., Stolberg, V.R., Hu, J.S., Komuniecki, E., and Chensue, S.W. (2004). The innate pulmonary granuloma: characterization and demonstration of dendritic cell recruitment and function. Am J Pathol *164*, 1021-1030.

Colley, D.G., and Secor, W.E. (2014). Immunology of human schistosomiasis. Parasite Immunol *36*, 347-357.

Costain, A.H., MacDonald, A.S., and Smits, H.H. (2018). Schistosome Egg Migration: Mechanisms, Pathogenesis and Host Immune Responses. Front Immunol *9*, 3042.

Cronan, M.R., Beerman, R.W., Rosenberg, A.F., Saelens, J.W., Johnson, M.G., Oehlers, S.H., Sisk, D.M., Jurcic Smith, K.L., Medvitz, N.A., Miller, S.E., *et al.* (2016). Macrophage Epithelial Reprogramming Underlies Mycobacterial Granuloma Formation and Promotes Infection. Immunity *45*, 861-876.

Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity *17*, 693-702.

Dewalick, S., Bexkens, M.L., van Balkom, B.W., Wu, Y.P., Smit, C.H., Hokke, C.H., de Groot, P.G., Heck, A.J., Tielens, A.G., and van Hellemond, J.J. (2011). The proteome of the insoluble Schistosoma mansoni eggshell skeleton. Int J Parasitol *41*, 523-532.

deWalick, S., Tielens, A.G., and van Hellemond, J.J. (2012). Schistosoma mansoni: the egg, biosynthesis of the shell and interaction with the host. Exp Parasitol *132*, 7-13.

Dunne, D.W., Hassounah, O., Musallam, R., Lucas, S., Pepys, M.B., Baltz, M., and Doenhoff, M. (1983). Mechanisms of Schistosoma mansoni egg excretion: parasitological observations in immunosuppressed mice reconstituted with immune serum. Parasite Immunol *5*, 47-60.

Freedman, D.O., and Ottesen, E.A. (1988). Eggs of Schistosoma mansoni stimulate endothelial cell proliferation in vitro. J Infect Dis *158*, 556-562.

Hams, E., Aviello, G., and Fallon, P.G. (2013). The schistosoma granuloma: friend or foe? Front Immunol *4*, 89.

Hutchison, H.S. (1928). The Pathology of Bilharziasis. Am J Pathol *4*, 1-16 11.

Jurberg, A.D., Goncalves, T., Costa, T.A., de Mattos, A.C., Pascarelli, B.M., de Manso, P.P., Ribeiro-Alves, M., Pelajo-Machado, M., Peralta, J.M., Coelho, P.M., *et al.* (2009). The embryonic development of Schistosoma mansoni eggs: proposal for a new staging system. Dev Genes Evol *219*, 219-234.

Klaver, E.J., Kuijk, L.M., Lindhorst, T.K., Cummings, R.D., and van Die, I. (2015). Schistosoma mansoni Soluble Egg Antigens Induce Expression of the Negative Regulators SOCS1 and SHP1 in Human Dendritic Cells via Interaction with the Mannose Receptor. PLoS One *10*, e0124089.

Mann, V.H., Suttiprapa, S., Rinaldi, G., and Brindley, P.J. (2011). Establishing transgenic schistosomes. PLoS Negl Trop Dis *5*, e1230.

Martinez., I.G. (1916). Investigations on the prevalence and clinical features of intestinal bilharziosis (Schistosomiasis monsoni) in Porto Rico. New Orleans Medical and Surgical Journal *69*, 352-394.

McManus, D.P., Dunne, D.W., Sacko, M., Utzinger, J., Vennervald, B.J., and Zhou, X.N. (2018). Schistosomiasis. Nat Rev Dis Primers *4*, 13.

Michaels, R.M., and Prata, A. (1968). Evolution and characteristics of Schistosoma mansoni eggs laid in vitro. J Parasitol *54*, 921-930.

Moore, D.L., Grove, D.I., and Warren, K.S. (1977). The Schistosoma mansoni egg granuloma: quantitation of cell populations. J Pathol *121*, 41-50.

Nation, C.S., Da'dara, A.A., Marchant, J.K., and Skelly, P.J. (2020). Schistosome migration in the definitive host. PLoS Negl Trop Dis *14*, e0007951.

Pagan, A.J., and Ramakrishnan, L. (2018). The Formation and Function of Granulomas. Annu Rev Immunol *36*, 639-665.

Pellegrino, J., Oliveira, C.A., Faria, J., and Cunha, A.S. (1962). New approach to the screening of drugs in experimental schistosomiasis mansoni in mice. Am J Trop Med Hyg *11*, 201-215.

Ramakrishnan, L. (2020). Mycobacterium tuberculosis pathogenicity viewed through the lens of molecular Koch's postulates. Curr Opin Microbiol *54*, 103-110.

Relman, D.A., Falkow, S., and Ramakrishnan, L. (2020). In A molecular perspective of microbial pathogenicity Mandell, Douglas and Bennett’s Principles and Practice of Infectious Diseases, Ninth Edition (Elsevier Inc.).

Schwartz, C., and Fallon, P.G. (2018). Schistosoma "Eggs-Iting" the Host: Granuloma Formation and Egg Excretion. Front Immunol *9*, 2492.

Smith, P., Fallon, R.E., Mangan, N.E., Walsh, C.M., Saraiva, M., Sayers, J.R., McKenzie, A.N., Alcami, A., and Fallon, P.G. (2005). Schistosoma mansoni secretes a chemokine binding protein with antiinflammatory activity. J Exp Med *202*, 1319-1325.

Swartz, J.M., Dyer, K.D., Cheever, A.W., Ramalingam, T., Pesnicak, L., Domachowske, J.B., Lee, J.J., Lee, N.A., Foster, P.S., Wynn, T.A., *et al.* (2006). Schistosoma mansoni infection in eosinophil lineage-ablated mice. Blood *108*, 2420-2427.

Takaki, K., Davis, J.M., Winglee, K., and Ramakrishnan, L. (2013). Evaluation of the pathogenesis and treatment of Mycobacterium marinum infection in zebrafish. Nature protocols *8*, 1114-1124.

Von, L. (1964). Studies on Granuloma Formation. Iii. Antigen Sequestration and Destruction in the Schistosome Pseudotubercle. Am J Pathol *45*, 75-94.

Von Lichtenberg, F., Erickson, D.G., and Sadun, E.H. (1973). Comparative histopathology of schistosome granulomas in the hamster. Am J Pathol *72*, 149-178.

Weinstock, J.V., and Boros, D.L. (1983). Modulation of granulomatous hypersensitivity. VI. T lymphocyte subsets influence mast cell density in liver granulomas of Schistosoma mansoni-infected mice. J Immunol *131*, 959-961.

WHO (2019). Schistosomiasis and soil transmitted helminthiases: numbers of people treated in 2018. Weekly Epidemiological Record *vol. 94, 50* 601–612.

Yang, C.T., Cambier, C.J., Davis, J.M., Hall, C.J., Crosier, P.S., and Ramakrishnan, L. (2012). Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. Cell Host Microbe *12*, 301-312.

FIGURE LEGENDS

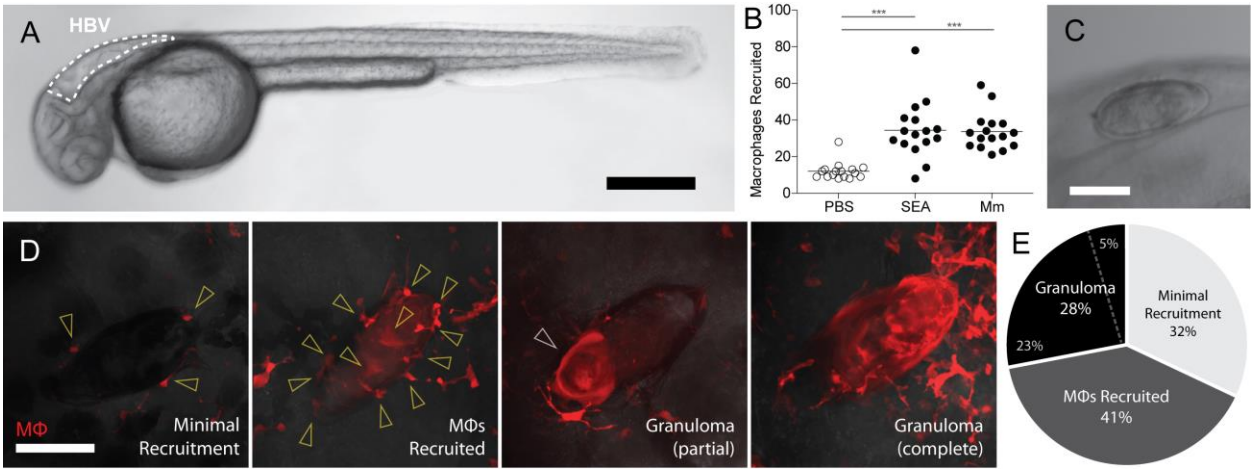


Figure 1. Macrophage responses to SEA and *S. mansoni* eggs

(A) Zebrafish larvae at 30 hpf with hindbrain ventricle (HBV) outlined. Scale bar, 300 μm. (**B**) Mean macrophage recruitment to HBV 3 hours post-injection with PBS, SEA or Mm; ANOVA with Dunnet's post-test. (**C**) *S. mansoni* egg in HBV immediately after implantation. Scale bar, 75 μm. (**D**) Representative images of macrophage responses to *S. mansoni* eggs observed 5 dpi; Minimal recruitment, few if any macrophages recruited with ≤6 in contact with the egg (arrowheads); Macrophages recruited, several macrophages recruited with >6 in contact with the egg (arrowheads) but without aggregation; Granuloma; macrophage aggregation in which individual macrophages cannot be distinguished as separate, either partially (arrowhead) or completely encasing the egg. Scale bar, 75 μm. (**E**) Prevalence of macrophage responses to implanted eggs as defined in **D**, representing 8 experiments, each constituting a separate batch of eggs and a separate clutch of zebrafish larvae, as detailed in **Table S1**. Dotted line divides the proportion of partial (23%) and complete (5%) granulomas. Also see Figure S1, Table S1 and Movie S1.

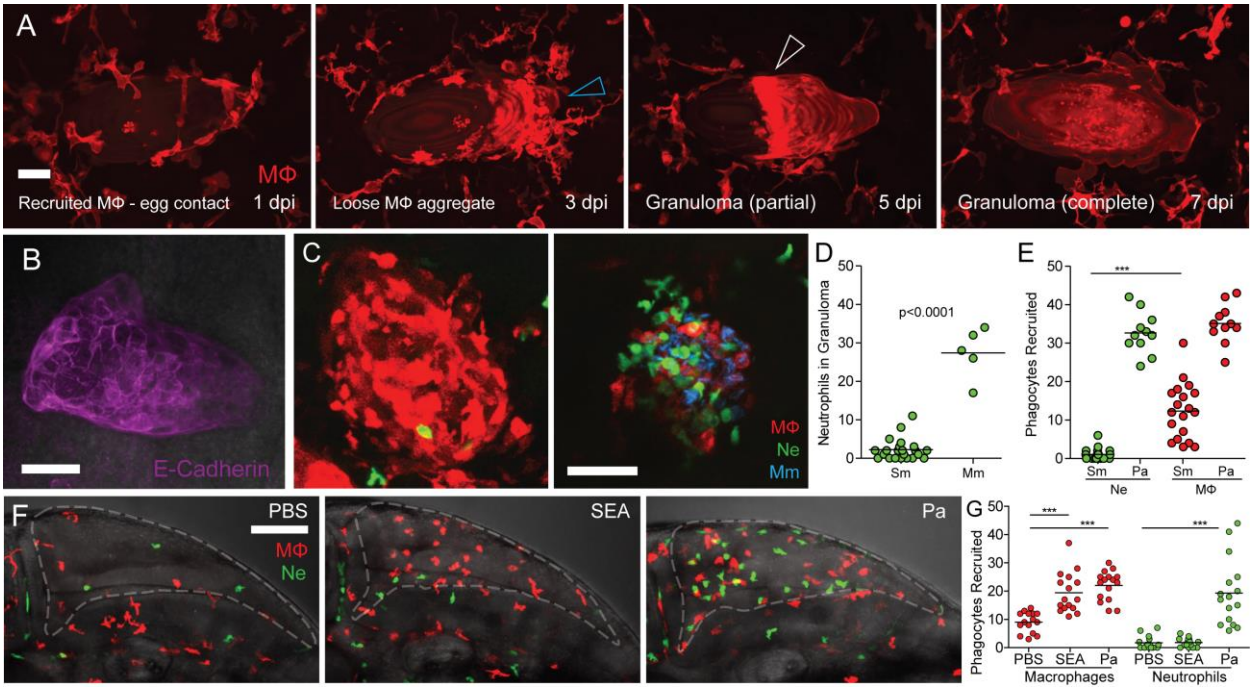


Figure 2. *S. mansoni* eggs induce epithelioid granulomas in larval zebrafish

(**A**) Timelapse microscopy of egg monitored at 2 day intervals from 1-7 dpi showing in the four panels, respectively, sequential macrophage recruitment, aggregation (blue arrowhead), formation of the partial granuloma (white arrowhead), and its expansion to encase the egg. Scale bar, 25 μm. (**B**) Epithelioid granuloma immunostained using E-cadherin antibody. Scale bar, 50 μm. (**C**) Confocal images of granulomas in representative transgenic zebrafish larvae with red-fluorescent macrophages (MΦ) and green-fluorescent neutrophils (Ne) at 5 dpi with *S. mansoni* eggs (Sm) (left), or 5 days post-infection with *M. marinum* (Mm) (right); Scale bar, 50 μm. (**D**) Quantification of neutrophils recruited to Sm and Mm granulomas. (**E**) Quantification of phagocytes recruited to Sm and *P. aeruginosa* (Pa) 6 hours post-injection. (**F**) Confocal images of HBV of representative larvae showing phagocyte recruitment at 6 hours post-injection with phosphate buffered saline (PBS) (left), *S. mansoni* soluble egg antigens (SEA) or *P.aeruginosa*. Scale bar, 100 μm. (**G**) Quantification of phagocytes recruited to Sm and *P.aeruginosa* (Pa) at 6 hours post-injection. Horizontal lines in (**D**,**E**,**G**) depict mean values. Student's t-test (**D**) or one-way ANOVA with Bonferroni's post-test (**E**,**G**). Experiments in (**A-B**,**E**) were done once each, those in (**C-D**,**F-G**) are representative of two experiments. Also see Figures S2 and S3 and Movie S2.

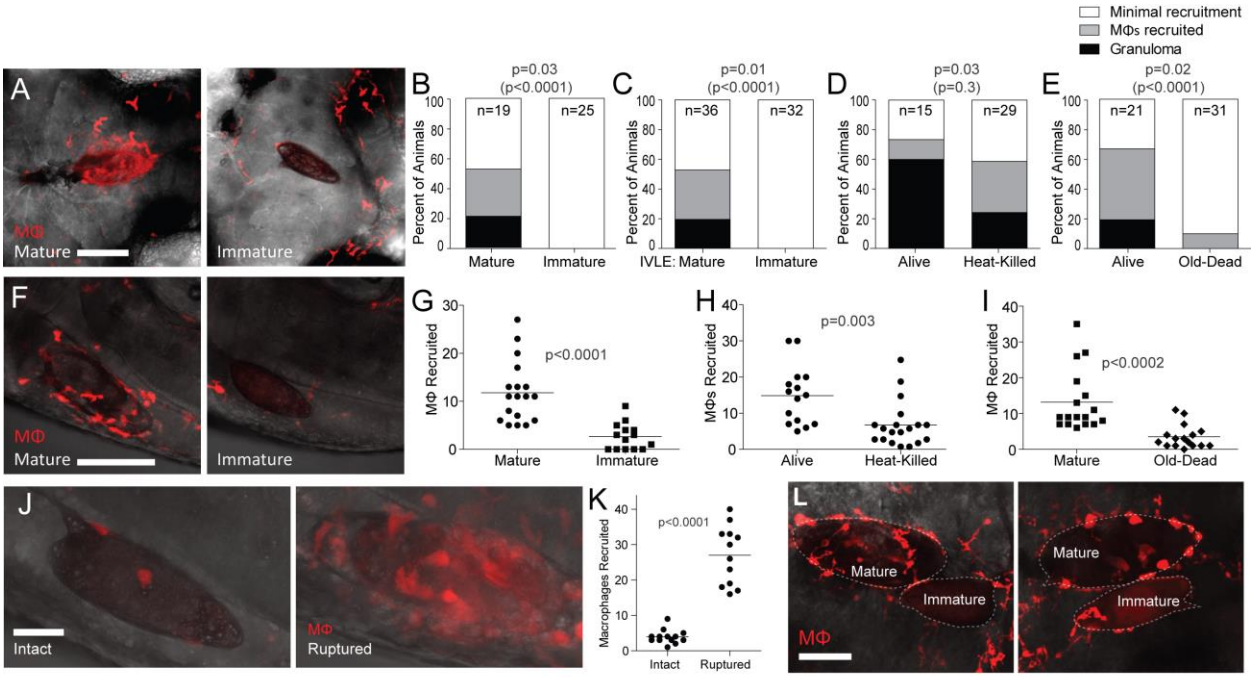


Figure 3. Immature eggs do not induce macrophage recruitment or granuloma formation

(**A**-**E**) Granuloma formation and macrophage recruitment at 5 dpi comparing mature eggs with (**A**,**B**) immature eggs, (**C**) immature IVLE, (**D**) heat-killed eggs, or (**E**) old-dead eggs. Representative images in (**A**), scale bar 100 μm. (**B**-**E**) Percent of animals with different levels of macrophage recruitment to the egg. (**F**-**I**) Macrophages recruited to mature eggs at 3 hpi compared to (**F** and **G**) immature eggs, (**H**) heat-killed eggs, and (**I**) old-dead eggs. Representative images in (**F**), scale bar, 100 μm. (**G**-**I**) Quantification of macrophages recruited. (**J**) Confocal images showing macrophage recruitment to intact and mechanically ruptured immature eggs 6 hpi. Scale bar, 25 μm. (**K**) Quantification of macrophage recruitment to intact and ruptured immature eggs 6 hpi. (**L**) Confocal images of macrophage recruitment 5 dpi to co-implanted mature and immature eggs into the same hindbrain ventricle of two different larvae. Enumeration of recruited macrophages showed 19 and 2 macrophages recruited respectively to the mature and immature egg (left panel), and 23 and 6 macrophages recruited respectively to the mature and immature egg (right panel). Scale bar, 50 μm. (**G**-**I**) Horizontal bars, mean values. Statistics, (**B**-**E**) Fisher's exact test comparing the proportion of eggs which induced granuloma formation (black bars), or

granuloma formation with macrophage recruitment (black and gray bars combined, in parentheses); (**G-I**, **K**) Student's t-test. (**B-E**) n, number of animals. All experiments performed once, except for **F**,**G**,**J**, and **K** which are representative of two experiments. Also see Figure S4 and Movie S3.

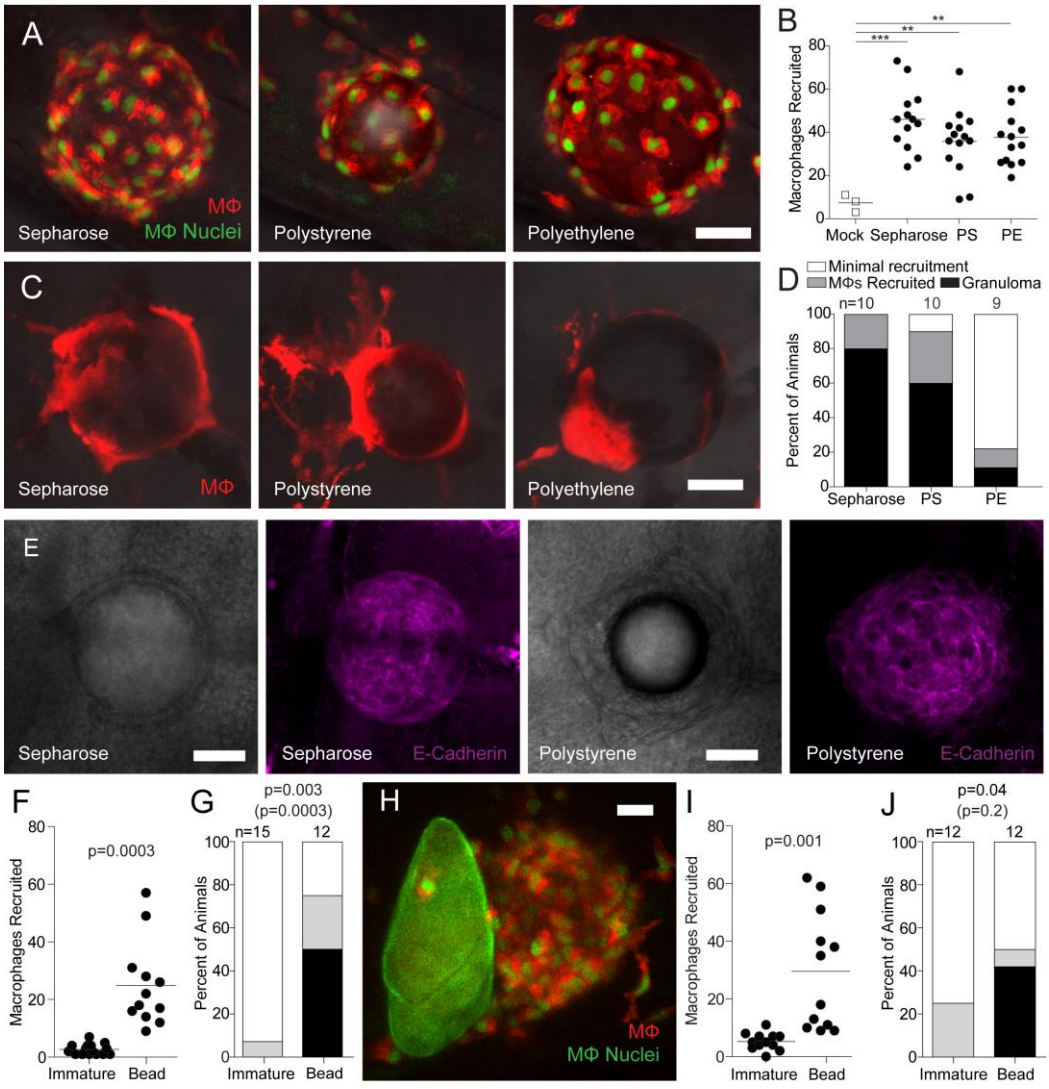


Figure 4. Chemically inert beads induce epithelioid granulomas

(**A**) Representative confocal images of macrophages recruited 6 hpi of sepharose, polystyrene or polyethylene microspheres into the HBV of transgenic zebrafish larvae carrying red-fluorescent macrophages with green nuclei. (**B**) Enumeration of macrophages recruited to these microspheres in multiple animals. (**C**) Representative confocal images of granulomas formed around the three types of

microspheres 5dpi into the HBV of transgenic larvae carrying the transgene for red-fluorescent macrophages (without green nuclei). (**D**) Stages of macrophage recruitment to microspheres 5 dpi into HBV of multiple larvae. (**E**) Brightfield (panels 1,3) and fluorescence confocal (panels 2,4) microscopy of sepharose and polystyrene bead granulomas following immunofluorescence staining with the E-cadherin antibody. (**F** and **G**) Macrophage recruitment to immature eggs or microspheres implanted into the HBV at 6 hpi (**F**) and 5 dpi (**G**). (**H**-**J**) Macrophage recruitment following co-implantation of an immature egg and a polystyrene microsphere into the HBV of larvae transgenic for red-fluorescent macrophages with green nuclei. (**H**) Representative confocal image of an immature egg next to a microsphere. (**I**,**J**) Quantification of macrophage recruitment at 6 hpi (**I**), and at 5 dpi (**J**). Scale bars, 25 μm. (**B**,**F**,**I**) Horizontal bars, means. (**D**,**G**,**J**) n, number of animals. Statistics, one-way ANOVA (**B**), unpaired (**F**) and paired (**I**) Student's t-test and Fisher's exact test comparing granulomas (black bars) or granuloma formation with macrophage recruitment (black and gray bars, in parentheses) (**G**-**J**). Experiments in **E** and **F**-**J** were performed once. **A**-**D** are representative of three experiments. Also see Table S2.

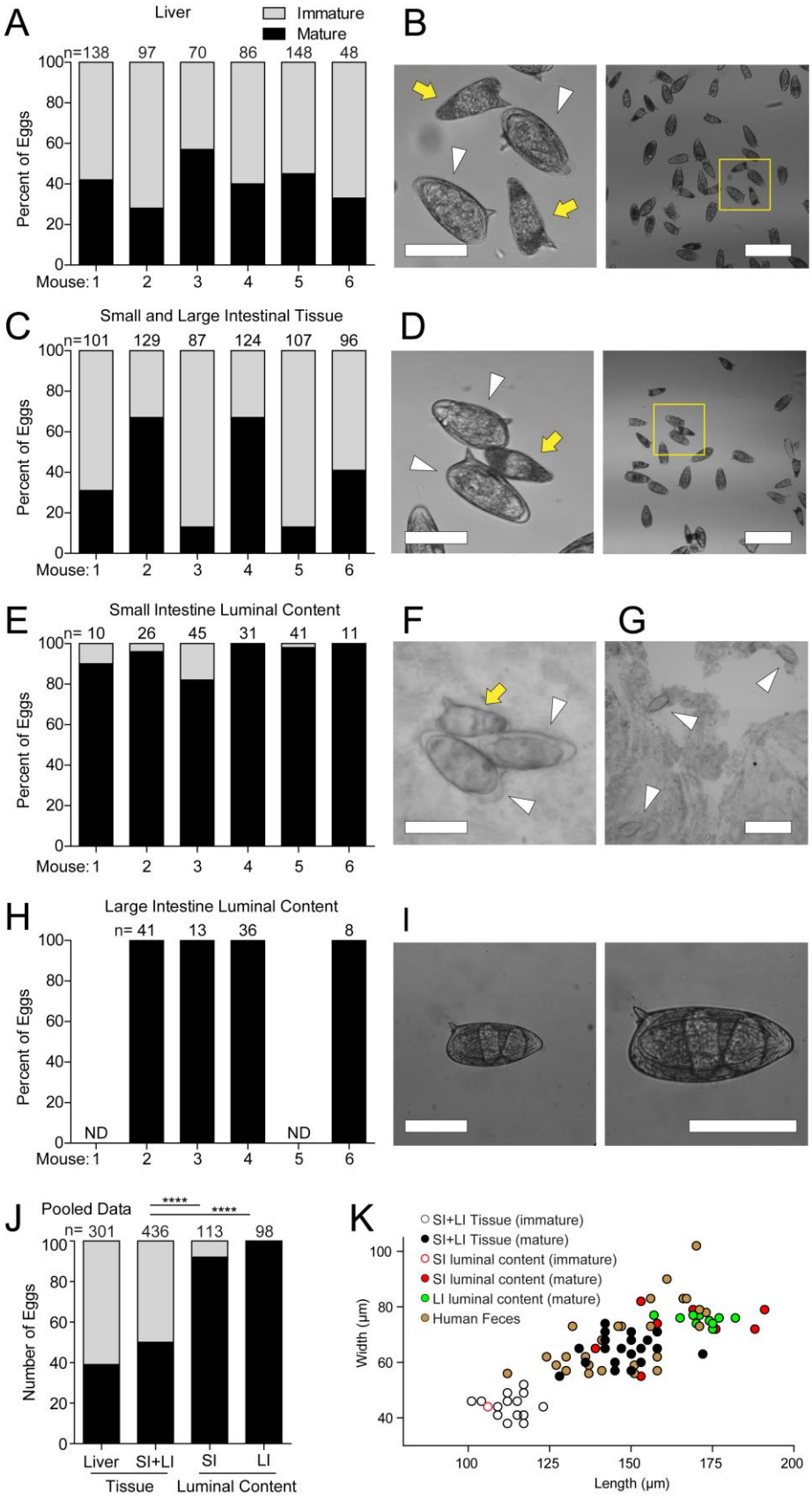

A
Liver
Immature
Mature
n=138 97 70 86 148 48
Percent of Eggs
Mouse: 1 2 3 4 5 6
B
C
Small and Large Intestinal Tissue
n=101 129 87 124 107 96
Percent of Eggs
Mouse: 1 2 3 4 5 6
D
E
Small Intestine Luminal Content
n= 10 26 45 31 41 11
Percent of Eggs
Mouse: 1 2 3 4 5 6
F
G
H
Large Intestine Luminal Content
n= 41 13 36 8
ND ND
Percent of Eggs
Mouse: 1 2 3 4 5 6
I
J
Pooled Data
****
****
n= 301 436 113 98
Number of Eggs
Liver SI+LI SI LI
Tissue
Luminal Content
K
SI+LI Tissue (immature)
SI+LI Tissue (mature)
SI luminal content (immature)
SI luminal content (mature)
LI luminal content (mature)
Human Feces
Width (µm)
Length (µm)

Figure 5. Mature eggs translocate into the lumen of the intestines

(**A-I**) Quantification (**A**,**C**,**E**,**H**) and representative brightfield images (**B**,**D**,**F**,**G**,**I**) of mature and immature eggs found in the liver (**A**,**B**), small and large intestinal wall tissue and vasculature (**C**,**D**), small intestinal luminal content (**E-G**) and large intestinal luminal content (**H**,**I**) for six individual *S. mansoni*-infected mice. (**B**,**D**) Representative images with image (left panel) showing immature (yellow arrow) and mature (white arrowhead) magnified from yellow square in wide-field image (right panel). (**F**,**G**) Images of eggs from the lumen of the small intestine, showing two mature eggs in contact with one immature egg (**F**), and a wide-field image showing three mature eggs (**G**). (**I**) Representative image of an egg recovered from feces at low resolution (left) and higher resolution with developed miracidia visible (right). (**J**) Pooled data for mice 2,3,4, and 6 from panels (**A**,**C**,**E**,**H)**. SI, small intestine; LI, large intestine. (**K**) Dimensions of eggs from this experiment which were classified as immature or mature (open or closed circles, respectively) plotted with eggs shed in the feces of *S. mansoni*-infected humans (Martinez., 1916). All scale bars 100 μm except for (**G**) and the right panels of (**B**,**D**) which are 300 μm. ND, not determined. Statistics, Fisher's exact test. Also see Figure S5.